Denaturation of hemoglobin under high pressure, II

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DENATURATION OF HEMOGLOBIN UNDER HIGH PRESSURE, II

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The denaturation process of carbonylhemoglobin under pressure of 0~7000 kg/cm² was examined kinetically at temperature of 10~75°C. The results are as follows: The process is of the first order kinetics. The temperature coefficient of the rate of denaturation is negative below about 30°C, while positive above that. At the region followed by thermal denaturation, that is, above about 70°C the rate is retarded by pressure up to about 2000 kg/cm² above which the rate increases again with the increase of pressure. These results are closely similar to those of ovalbumin[2].

In part I[1] the qualitative properties of hemoglobin under high pressure have been discussed mainly on oxyhemoglobin, but kinetic considerations have not been done because of the complexity of the oxidation of the heme part.

In this paper the denaturation process of carbonylhemoglobin under pressure was examined kinetically. Carbonylhemoglobin is not oxidized when its globin part is denatured, though it has a structure similar to oxyhemoglobin, and the process of denaturation follows the simple first order kinetics which enables us to make the kinetic analysis.

To compare the data with those of ovalbumin[2], the rates of coagulation were measured under pressure of 0~7000 kg/cm² and at temperature of 10~75°C.

Experimentals

Carbonylhemoglobin was prepared by the similar method of Adair and Adair[3], recrystalized three times and dialyzed. A part of experiments was carried out using a hemolyzed suspension of well carbonylated red cell, but there was no detectable difference in the behavior observed.

The concentration of carbonylhemoglobin in the test sample was about 0.5%, and the pH of the solution was adjusted to 6.8 with sodium phosphate dibasic and potassium phosphate monobasic. The concentration of phosphate was always adjusted to M/40. Cares were needed to avoid oxygen contamination.

The compressing apparatus and its procedures were the same as in the previous papers[1,2].

Results

1) K. Suzuki and K. Kitamura, *This Journal*, 29, 81 (1959)
2) K. Suzuki, *ibid.*, 28, 24 (1958)
3) G. S. Adair and M. E. Adair, *Biochem. J.*, 28, 1231 (1934)

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The color of coagulated protein from carbonylhemoglobin is pink red in the isoelectric solution. It becomes soon brownish when exposed to air. The protein in the filtrate seems to be native carbonylhemoglobin: its absorption spectra[4] in the wave length between 500 and 600 mμ is not changed from that of the original test sample.

In Fig. 1 are shown the semilogarithmic plots of the protein concentration of the filtrate

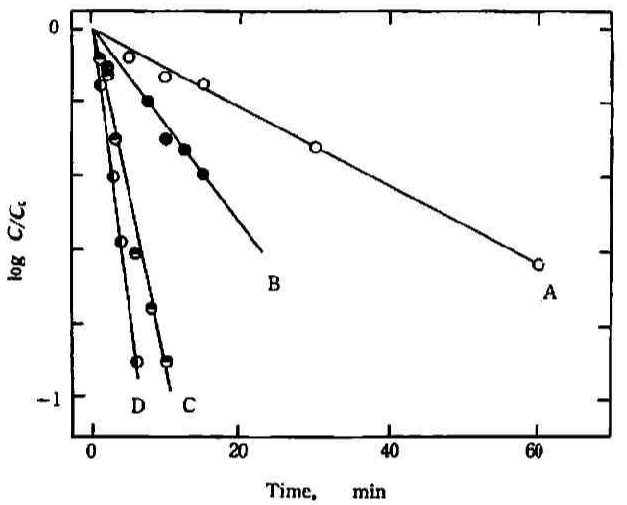

Fig. 1 Relations between logarithm of C/C_0 and pressing duration (C is the protein concentration in filtrate and C_0 is the initial protein concentration.)

buffer: M/40 phosphate buffer, pH 6.8
temperature: 24°C

	pressure, kg/cm²
A:	6000
B:	6500
C:	6800
D:	7000

against duration of compression. The linear relationships are found in the early stages, and so the process is of the first order with regard to the protein concentration. But, as the concentration of the filtrate decreases to about 1/10 of the beginning, the plots deviate from a linear relationship. This is maybe due to the renaturation of carbonylhemoglobin from coagulated hemochrome.

The effects of pressure and temperature are surveyed in Fig. 2. It is observed from the figure that under several thousands kg/cm² of pressure carbonylhemoglobin is denatured in the

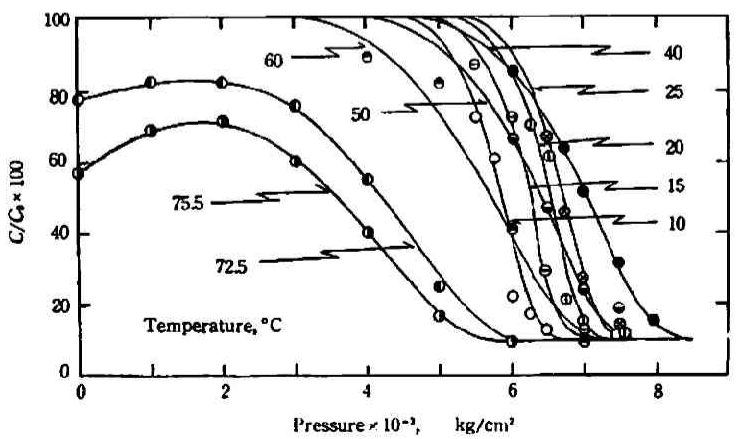

Fig. 2 Relations between $C/C_0 \times 100$ and pressuer

buffer: M/40 phosphate buffer, pH 6.8
duration: 5 min

4) M. Suzuki, A. Kajita and C. Hanaoka, *J. Biochem.*, 41, 401 (1954)

The Review of Physical Chemistry of Japan Vol. 29 No. 2 (1959)

whole range of temperature examined, and there is a minimum in rate between 30 and 40°C. With the increase of pressure, most of protein coagulate, but about 1/10~1/20 of the beginning remains intact even though at very high pressure. The incompleteness of coagulation of hemoglobin thus observed even under very high magnitude of pressure and very long duration of compression may be ascribed to reversibility of denaturation, as are described already in part I[1]. At the higher temperature, where heat denaturation occurs under ordinary pressure, it is observed that the application of pressure retards the rate of coagulation up to about 2000 kg/cm², above which the rate increases again with the increase of pressure.

Discussions

As shown in Fig. 1, the process of denaturation being of the first order, the rate constant k is calculated from the following equation,

$$k = \frac{1}{t} \ln(C_0/C),$$

where t is the duration of compression, and C_0 and C are the initial and the final (after the lapse of time t) concentrations of carbonylhemoglobin in the solution respectively.

The influence of pressure on the rate is related by the equation,

$$\frac{d \ln k}{dP} = -\frac{\Delta V^{\ddagger}}{RT},$$

where P is the magnitude of pressure. R is the gas constant, T is the absolute temperature and $\Delta V^{\ddagger}$ is the molar volume change of activation. Accordingly, the semilogarithmic plots of the rate constants against pressure afford the value of $\Delta V^{\ddagger}$ (*cf.* Fig. 3). As shown in Table 1, the values of $\Delta V^{\ddagger}$ under very high pressure (about 4000 kg/cm²) are negative over the whole temperature range examined, but their absolute values decrease as the temperature increases.

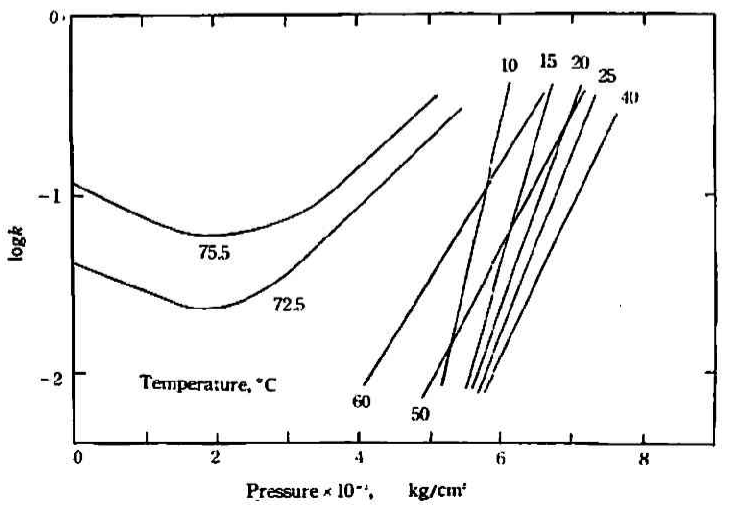

Fig. 3 Relations between logarithm of rate constant, k (min⁻¹) and pressure

Table 1 Molar volume change of activation, $\Delta V^{\ddagger}$, cc/mole

Temperature, °C	10	15	20	25	50	60	72.5	75.5
$\Delta V^{\ddagger}$ >4000kg/cm²	−99	−85	−68	−68	−47	−44	−25	−24
$\Delta V^{\ddagger}$ 0～1000kg/cm²							+17	+25

Corresponding to the retardation effect of pressure in thermal denaturation, the values of $\Delta V^{\ddagger}$ at the higher temperature are positive below about 2000kg/cm².

The studies on the temperature dependence of rate constant give the values of apparent activation energy, E from the relations between the logarithm of rate constant and the reciprocal of the absolute temperature. The plots are shown in Fig. 4, which have minima around 30～40°C, and are curved in the side of the lower temperature instead of a linear relationship. The values of E calculated are shown in Table 2. From these results it is to be noted that E is positive above 40°C, while negative and dependent on temperature below 30°C.

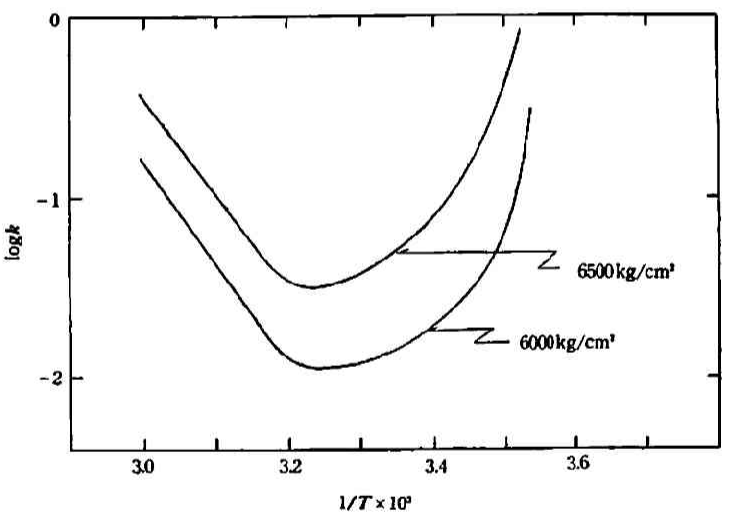

Fig. 4 Relations between logarithm of rate constant, k (min^{-1}) and reciprocal of absolute temperature, T

Table 2 Apparent activation energy, E

Pressure, kg/cm²	Activation energy, E, kcal/mole				
	10°C	15°C	20°C	25°C	40～75.5°C
6000	−29	−20	−17	−12	+25
6500	—	−29	−21	−21	+24

72.5～75.5°C

Pressure, kg/cm²	0	1000	2000	3000	4000	5000
E, kcal/mole	80	73	71	57	42	38

Regardless of the slight differences, it is possible to say that the characteristics of carbonylhemoglobin are similar to those of ovalbumin. It is, therefore, postulated that the behaviors found in these proteins may be due to the common denaturation mechanisms and the common structures in protein molecules under pressure.

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